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STUDIES ON TYPHUS AND SPOTTED FEVER

ANNUAL PROGRESS REPORT

by

Charles L. Wisseman, Jr., M.D.

April 1977
(For the period 1 July 1975 to 30 June 1976)

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND (SGRD-RP)
WASHINGTON, DC 20314

Contract No. DADA 17-71-C-1007

University of Maryland School of Medicine
Department of Microbiology
Baltimore, Maryland 21201

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Methodology has been developed for detection of antibiotic resistant mutants of R. prowazeki, determination of mutant frequencies and possibly mutation rates. A mouse test for virulence of R. prowazeki has been refined. Search for transformants resulting from selective intracellular release of DNA is in progress. Growth characteristics of R. mooseri differ from those of R. prowazeki.

Fifteen strains, 8 established species and 7 field isolates, of spotted fever

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered) group rickettsiae have been subjected to intensive morphological, biological, and serological study. Certain properties are held in common by most of the strains (slime layer, type 2 growth cycle, large plaque size, doxycycline sensitivity, penicillin-induced spheroplast formation) whereas serological characteristics appear to segregate the strains into species and sub-groups of related strains.

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SUMMARY

Studies on virulent and attenuated *Rickettsia prowazeki*. Plaque-purified, working seeds of Ery^{r+} E strain and the Pen^{r+} Breinl strain of *R. prowazeki* were quantitatively characterized with respect to antibiotic resistance, uptake characteristics and intracellular growth rate. This information, applied to our standardized systems, permitted us to attempt intracellular transformation through predictable infection of host cells with both organisms and selective intracellular lysis of the Ery^{r+} strain with penicillin with liberation of DNA into host cell cytoplasm in the presence of intact Pen^{r+} organisms. Search for transformants, still incomplete, has not yet yielded dually resistant organisms. Systemic studies of the problems have led to improved methods for detection of mutants and determination of mutant frequencies and have opened the possibility of a practical method for determination of mutation rates. A mouse system for measuring *R. prowazeki* virulence has been improved to a workable level.

R. prowazeki has been found to undergo limited multiplication in enucleated host cells and human lymphocytes. An external CO_2 source appears to be required for growth. *R. mooseri* was found to have a Type 2 (*R. rickettsii* type), spreading infection cycle in contrast to *R. prowazeki*. Fresh, wild, low-passage strains of *R. prowazeki* and of *R. mooseri* have been isolated directly in CE cell cultures from blood of Burundi typhus patients and kidneys of naturally-infected rats from Ethiopia, respectively.

Studies on Spotted Fever Group rickettsiae. Eight established prototype strains and 7 field isolates (Pakistan, Thailand, Czechoslovakia, Israel) have been plaque purified and characterized according to several new properties. All strains, except *R. akari* (incomplete) possess a slime layer or capsule, exhibit a Type 2 (spreading) infection cycle in cell cultures, are inhibited by doxycycline at 0.1 µg/ml and form intracellular spheroplasts in the presence of penicillin G (100 µg/ml). Under highly standardized conditions, all strains, except *R. akari* and *R. australis*, form large ($\sim$1mm) plaques. *R. akari* and *R. australis* form somewhat smaller plaques. Stable plaque size variants were not encountered. A method for preparing substantial quantities of reasonably specific immune ascitic fluid in mice holds considerable promise. Preliminary studies with mouse sera in the microagglutination test suggest that some field isolates are related to established strains (variants within a group) whereas others appear to be very different and may constitute valid new species.

RESEARCH PROGRESS

A. Studies on Virulent and Attenuated *Rickettsia prowazeki*.

1. Genetics, genome and markers. Since the "state of the art" of quantitative methodology for genetic studies with rickettsiae is still very primitive and the inherent characteristics of the systems pose some problems and limitations, much effort this past year has been devoted to attempting to adapt recent methodological advances in rickettsiology to the detection and quantitation of mutants and attempts to transfer DNA by transformation, both using antibiotic resistance markers as convenient models, and the search for virulence markers. Definite progress has been made, though at a rate slower than hoped for because of certain problems inherent in the rickettsia-host cell system (*vide infra*).

a. Characteristics of antibiotic resistant mutant strains of *R. prowazeki*. In preparation for transformation experiments and other studies (*vide infra*), large seeds of the plaque purified erythromycin resistant (Ery^{r+}) mutant of the attenuated E strain and of the penicillin resistant (Pen^{r+}) mutant of the virulent Breinl strain of *R. prowazeki* were prepared by growing in CE cell cultures to an estimated late log phase of growth.

Uptake and growth characteristics of the two seeds were carefully determined. The quantitative relationships previously established permitted (i) the construction of simple graphs which would permit quick reading of the dilution of each seed required to give any desired % cells infected or average number of rickettsiae per cell with CE cells in our standard suspended cell infection system and (ii) estimate the incubation period, under our standard conditions, required to achieve any desired average number of rickettsiae per infected cell, given the average number of ricketsiae per infected cell at time of infection. These relationships proved practically reproducible and permitted us to conduct the intracellular transformation experiments described below with the required precision for the predictable introduction and manipulation of each strain in dually infected cells. We believe that this is a substantial advance in quantitative methodology for rickettsiae and fully justifies the tedious antecedent studies of basic rickettsia-host cell interactions which laid the foundations for this.

Full scale plaque reduction tests were performed with penicillin G and erythromycin for both the Pen^{r+} and the Ery^{r+} strains. Table 1 records the results. The MIC for penicillin G was found to be 20 μg/ml for the Ery^{r+} strain, similar to the wild Breinl strain from which the Pen^{r+} strain was derived, and 600 μg/ml for the Pen^{r+} strain. The MIC for erythromycin was 0.03 μg/ml for the Pen^{r+} strain and greater than 100 μg/ml for the Ery^{r+} strain.

Figure 1. Plaque-reduction sensitivities of the penicillin resistant Breinl strain and the erythromycin resistant E strain of _R. prowazeki_ to penicillin G and erythromycin.

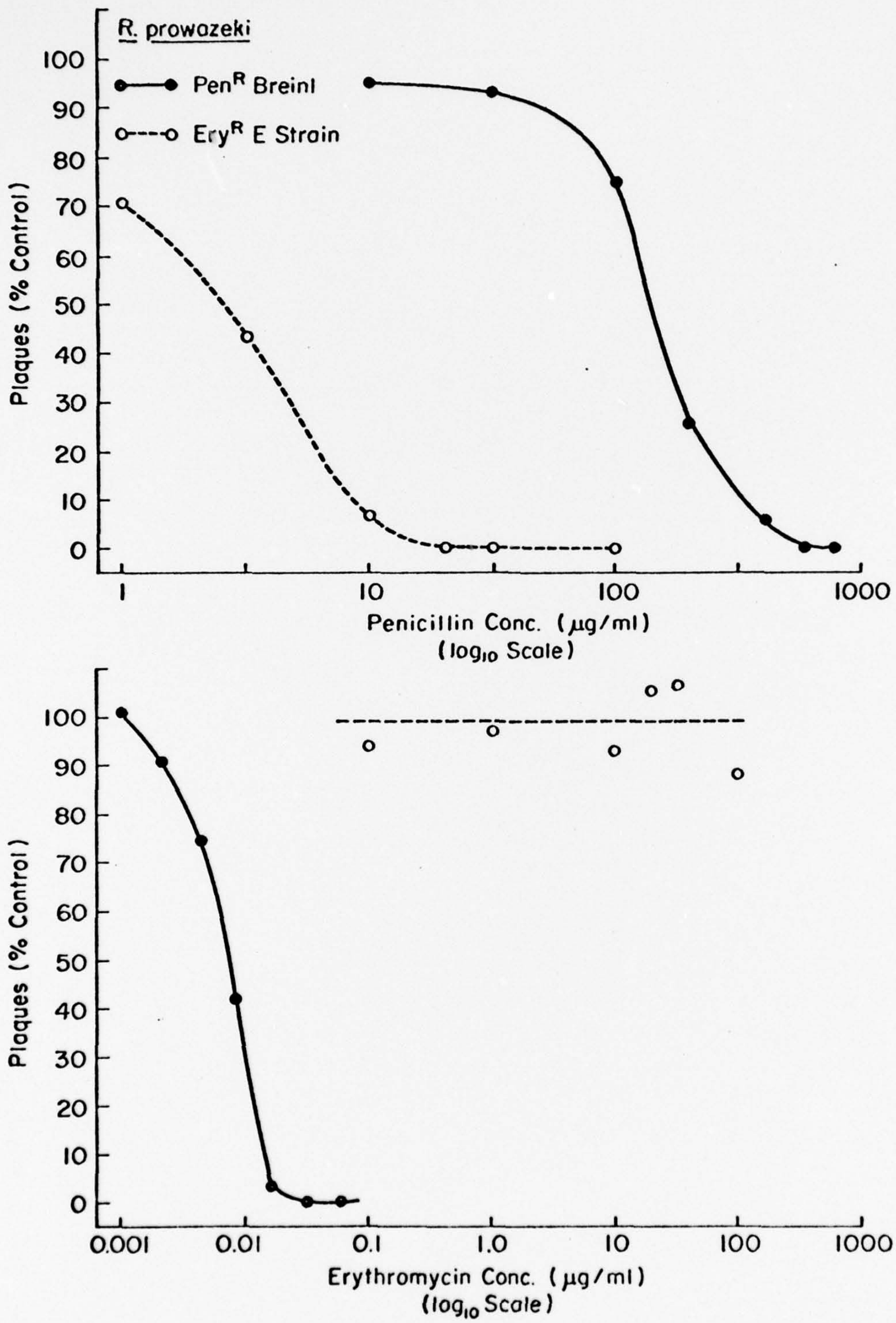


Figure 2. Suspended cell dose-response uptake kinetics of working seeds of the Pen^{r+} Breinl strain and the Ery^{r+} E strain of R. prowazeki. These relationships permit one to select the appropriate dilution of seed to obtain any desired N (average number of rickettsiae per cell) with each strain. Since, within limits, the two strains behave independently in mixtures, it is possible to infect host cells with both strains in desired proportions. A similar graph was constructed in which percent cells infected could be pre-selected under standard conditions.

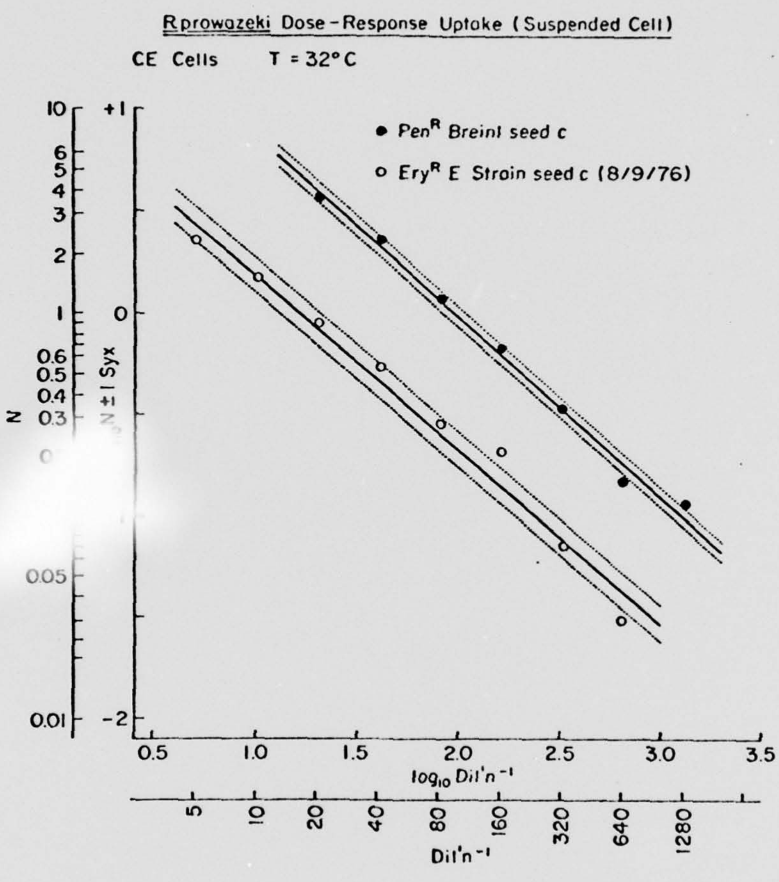
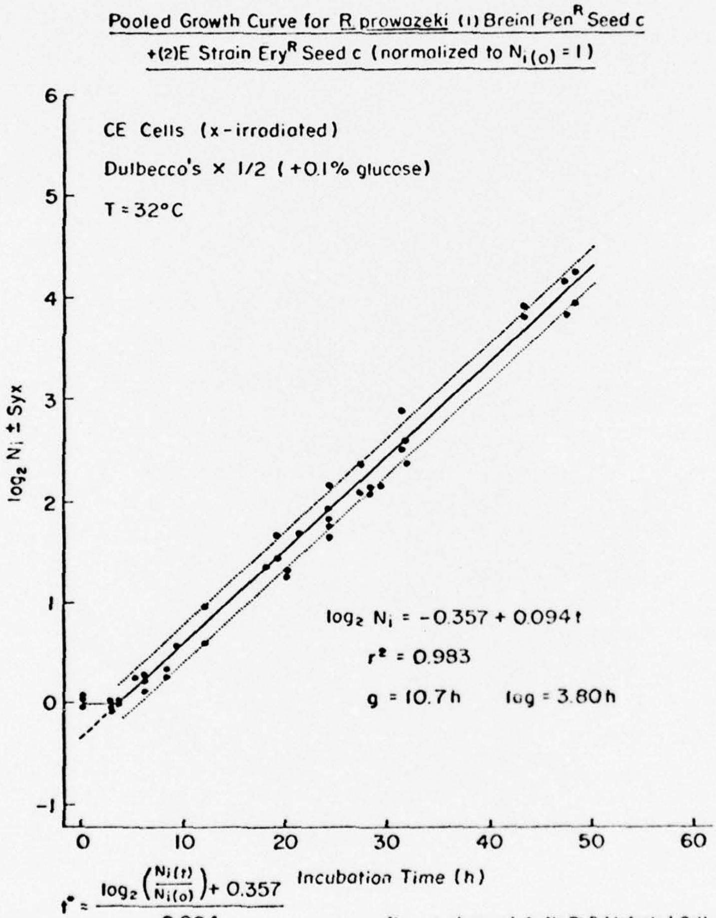


Figure 3. Growth kinetics of Pen^{r+} Breinl strain and Ery^{r+} E strain of R. prowazeki in checken embryo cells. Growth of the two strains was indistinguishable. Knowing the average number of each rickettsia per cell (See Fig. 2), it is possible to determine the incubation time necessary to achieve a desired intracellular population of each strain. Each control of the system is necessary to perform precise experiments on intracellular transformation.



$$t^{*} = \frac{\log_2\left(\frac{N_{i(t)}}{N_{i(o)}}\right) + 0.357}{0.094}$$

$N_{i(o)}$ = observed Av No RLB / Infected Cell

$N_{i(t)}$ = desired Av No RLB / Infected Cell

t = hrs to $N_{i(t)}$

*between 4 + 48h

Table 1. Some properties of antibiotic resistant mutants of R. prowazeki.

	R. prowzeki Pen^{R+} Breinl	Ery^{R+} E Strain
Minimal inhibitory Conc.		
Penicillin G (μg/ml)	600	20
Erythromycin (μg/ml)	0.03	>100
Mutant Frequency for Resistance to		
Pencillin G (100 μg/ml)		$<10^{-5.3}$
Erythromycin (1.0 μg/ml)	10^{-6}	

In order to be able to discriminate between spontaneous mutation and transformation in experiments described below, it is essential to have knowledge of the frequency of mutants resistant to the other antibiotic for each of the resistant strains. Neither strain showed a mutant frequency greater than 10^{-5} - 10^{-6} (Table 1), the limit of the direct method (*vide infra* for description of methods and problems).

b. Methods for detecting and quantitating *R. prowazeki* mutants. The capacity to detect mutants and to quantitate mutant frequencies and mutation rates is essential to the study of genetic phenomena in rickettsiae. We have chosen our standard CE cell plaque system and antibiotic resistance markers as a convenient and useful model amenable to quantitation for exploring methods and have employed the parent Pen^{R-} Ery^{R-} Dox and the mutant Pen^{R+} Ery^{R-} Dox^{R-} lines of the virulent Breinl strain and the Pen^{R-} Ery^{R+} Dox^{R-} mutant of the attenuated E strain of *R. prowazeki* in a variety of experiments. We have ascertained that there are certain inherent limits in this system, detected certain as yet not fully explained problems and devised compromise methods to circumvent some of the limitations.

The confluent CE cell monolayers in 60 mm petri dishes employed for plaquing contain about 10^7 cells. With ordinary overlay, 2-3 x 10^2 discrete plaques can be formed but as the number of PFU approaches 5 x 10^2 to 10^3, the monolayers show confluent lysis. When increasing numbers of PFU of a sensitive strain are introduced into plates with an overlay that contains 100 μg/ml penicillin G, which is several fold the MIC and completely inhibits rickettsial growth and is relatively non-toxic for the host cells, up to 10^5 PFU, enough to infect 1% of the host cells, can often be introduced into a plate without cell lysis, though even under these conditions some lysis does occur from time to time. At inputs of 10^6 PFU or greater, lysis of the monolayers occurs regularly. If 1.0 μg/ml doxycycline (10-fold the MIC), which does not lyse host cells alone, is substituted for penicillin, lysis of cell sheets sometimes occurs at rickettsial inputs of 10^3 to 10^4 PFU per plate. The reasons for this are not known, but it is obvious that mutant frequencies of less than 10^{-5} at best, and in some instances less than 10^{-3} cannot be detected and quantitated - seriously limiting this potentially powerful tool. Furthermore, when doxycycline concentrations only 2-3 x the MIC were employed, small numbers of plaques were not detected with large inputs as anticipated, suggesting that there may be some non-antibiotic inhibition of plaque formation.

More recent experiments with doxycycline have been somewhat more successful. Although lysis of cell sheets remains a problem, some more satisfactory experiments have been performed at high rickettsial inputs and what appear to be faint, very small plaques have been observed at a concentration of doxycycline of 1.0 µg/ml (10 times the MIC) with a frequency between 10^{-4} and 10^{-5}. If, on picking, amplifying and characterizing, these "plaques" are indeed caused by doxycycline resistant R. prowazeki, the practical implications are enormous because, as we have previously reported, conditions conducive to selection of doxycycline resistant strains of R. prowazeki in "nature" exist in the African scene (see Am. J. Epidemiol. 98: 262-282, 1973) -- a doxycycline resistant mutant frequency of 10^{-4} or 10^{-5} in a louse with 10^8 + organisms ingesting doxycycline - containing blood! There are reasons, however, to proceed with caution until definitive experiments have been done to conform this very preliminary observation. The "plaques" are atypical; even though we use SPAFAS eggs for these studies, we have previously encountered extraneous "plaques" in this system and have, from conventional eggs, isolated a doxycycline-resistant, non-rickettsial small plaque-forming bacillus that stains with Gimenez strain, etc. So, until we can isolate these "plaque-forming" agents and demonstrate them to be doxycycline-resistant R. prowazeki, we cannot be absolutely certain that we have demonstrated a high frequency of doxycycline resistant mutants.

Using the Penicillin - Erythromycin systems, experiments were performed to determine if a small number of resistant organisms could be detected in the presence of a large number of sensitive organisms. Thus, when approximately 20 PFU of the Pen^{R+} Ery^{R-} Dox^{R-} Breinl strain mutants were introduced into plaque dishes in a mixture with about 10^5 PFU of the Pen^{R-} Ery^{R+} Dox^{R-} E strain mutant under an agarose overlay containing 100 µg/ml penicillin G, very nearly the expected Pen^{R+} plaques were detected. When the ratios of the strains were reversed and 1.0 µg/ml erythromycin was included in the overlay, about half the expected number of Ery^{R+} plaques were detected.

Thus, in the Pen-Ery system, a small number of resistant PFU can be detected in the presence of up to 10^5 sensitive PFU. No explanation, however, is at hand yet to explain why lysis occurs with an input of about 10^6 PFU when this represents infection of only 10% of the available CE cells with non-replicating organisms. These studies do substantiate the view that in the Pen and Ery systems, mutant frequencies as low as 10^{-5} can be detected. However, since mutant frequencies in bacteria are often between 10^{-7} and 10^{-9}, or lower, the 10^{-5} limit is a severe restriction.

One method for possibly circumventing the 10^{-5} limit in non-toxic systems as Pen and higher limits as in Dox is currently under investigation. Although it entails a substantial loss in quantitative precision, its sensitivity may approach 10^{-8}. It involves introducing up to 10^5 infected cells into a plate with the usual 10^7 uninfected cells, allowing the rickettsia to grow uninhibited to near the end of the first infection cycle when each cell should have as many as 10^3 organisms, making the total number of organisms in the plate approach 10^8 in 10^5 discrete bundles, and then adding the antibiotic or other selective agent. Some preliminary experiments suggest that this method is feasible and will yield discrete plaques.

The delayed addition of antibiotic described above would no longer be a method for strict measurement of mutant frequency, since any excess resistant plaques would have had to arise by mutation during rickettsial growth. Indeed, the method may turn out to be better adapted for the measurement of mutation rate. Thus, although the development of methodology has been slow and beset with limitations on sensitivity and with a number of technical problems, steady improvement is occurring and systems workable within certain limits are evolving so that phenomena of both basic and very practical importance can be studied quantitatively.

c. Attempts to induce intracellular transformation. In these studies, CE cells dually infected with both the Pen^{R+} Ery^{R-} E strain mutant and the Pen^{R-} Ery^{R+} Breinl strain mutant were incubated until the rickettsial populations had ttained the desired levels. Then 100 µg/ml penicillin G was added to induce unstable spheroplast formation in the Pen^{R-} Ery^{R+} E strain mutant with liberation of its DNA in the host cell cytoplasm in the presence of intact Pen^{R+} Ery^{R-} rickettsiae. Finally, erythromycin (1.0 µg/ml) was added to allow only transformed Pen^{R+} Ery^{R+} Breinl strain rickettsiae, if present, to grow. These studies require an extraordinary amount of quantitative predictability on production of dually infected cells, growth rates of the two strains, kinetics of spheroplast formation and disruption and capacity to detect dually resistant transformed mutants. However, the systems and basic information have been worked out previously (earlier reports and (a) and (b) above) and microscopic examination of parallel slide chamber cultures subjected simultaneously to the same sequence of manipulations indicated that we can, and have on several occasions, achieved the desired intracellular manipulations of the two strains in dually infected cells with the production of unstable spheroplasts in cells also containing intact Pen^{R+} organisms.

Two systems have been used to carry out these manipulations and to search for transformants. Both systems begin with the production of cells infected with both strains in the suspended cell system. The % cells infected and average number of infected cells are determined. (i) The first system consists of introducing the infected cells into tissue culture flasks under fluid medium, incubating them until the desired intracellular populations have been attained, adding the penicillin to the fluid overlay, incubating until a high proportion of the resulting spheroplasts have disrupted and then adding erythromycin. Further incubation should allow dually resistant transformants to grow. Then the cells are harvested, disrupted and a "seed" is prepared. This material is examined directly and after further amplification in cell cultures containing both penicillin and erythromycin for dually resistant transformants by plaquing under agarose overlay containing penicillin, erythromycin or both penicillin and erythromycin.

(ii) The second method is carried out entirely in plastic petri dishes. About 10^5 dually infected cells are introduced into a petri dish with 10^7 uninfected CE cells and a non-inhibitory agarose overlay is added. After incubation to permit rickettsial growth, a penicillin overlay is added, followed by furthur incubation to allow spheroplast formation and disruption. Then erythromycin is added and the plates are incubated for plaque-formation. A plaque should form at the site of any cell in which transformation with dual resistance has taken place in at least one rickettsia. Plaques are counted, picked, amplified and tested for antibiotic resistance characteristics.

Several experiments have been carried out in which the desired intracellular events have taken place. We are now in the tedious process of examining the rickettsiae from these experiments for doubly resistant organisms. To date we have not found evidence for transformation but the examination of progeny is far from complete.

d. Tests for virulence. Ultimately, we intend to examine the E strain for stability of its attenuation to determine the risk of virulent back-mutation, making use of the principles established with the antibiotic models. A convenient laboratory correlate of virulence is essential to such a study. Extensive survey of host cells has failed to reveal one which shows the capacity to discriminate between virulent and attenuated *R. prowazeki*, except the cumbersome *in vitro* human macrophage system previously described. Accordingly we have refined the previously known mouse system, which makes use of differences between the attenuated E strain and the virulent Breinl strain to induce antitoxic immunity following intraperitoneal inoculation. Thus, by titration - toxic back-challenge methods, it is possible to determine the number of PFU of each rickettsial strain in 1 toxic PD_{50} dose. The number of virulent Breinl strain PFU in one toxic PD_{50} dose was found to be of the order of $10^{2.4}$ whereas, with the attenuated E strain, it was approximately $10^{5.3}$. Thus, it requires approximately 1,000 times as many PFU of the E strain as of the Breinl strain to protect 50% of the mice against 2 toxic LD_{50}. This difference is sufficiently large to serve as a useful correlate of virulence. Though somewhat cumbersome, it should allow us to achieve our objectives.

e. The genome of *R. prowazeki*. The studies described above have required such a large proportion of the laboratory effort that very little progress has been made in the past few months on the characterization of the *R. prowazeki* genome. However, using ethidium bromide and the "DAPI" reagent, and examining under the fluorescence microscope it has been possible to stain an intracellular structure within the rickettsiae which resembles that described as the nucleoid by others for bacteria. The size is small, near the limits of the optical microscope.

2. Biology of typhus group rickettsiae. Limited studies have continued on the biology of the typhus rickettsiae in their host cell interactions. The capacity of *R. prowazeki* to undergo limited multiplication in enucleated CE and L cells suggests that the host cell nucleus need not be continuously present for intracytoplasmic growth. *R. prowazeki* has been shown to be able to infect and grow in human peripheral lymphocytes in culture. Some evidence is accumulating to indicate that *R. prowazeki* requires an external source of CO_2 for growth. Surprisingly, *R. mooseri* has been found to exhibit a spreading, type 2, infection cycle similar to that of the spotted fever group rickettsiae (see section on SF group rickettsiae).

Significantly, R. prowazeki has been isolated from acute phase human blood (Burundi typhus patients) by plating directly onto CE monolayers and examining for plaques. Plaques have been picked and are being established as freshly isolated wild virulent strains of low passage level.

Some progress has been made towards growing R. prowazeki in high density (10^8 cells/ml) CE suspended cell cultures. Refinements are necessary to develop the method to a practical reliable method but the prospects seem reasonably promising that this may permit us to produce substantial quantities of cell culture-grown R. prowazeki in any desired growth phase.

B. Studies on Spotted Fever Group Rickttsiae. Spotted fever group rickettsial isolates from Pakistan, Thailand, Czechoslovakia and Israel were compared with established species in a systematic study employing new information on ultrastructure, growth cycle, serology and antibiotic sensitivity in order (1) to establish group characteristics and (2) to identify species characteristics.

1. Strains and plaque purification. Strains selected for detailed study are listed in Table 2, although some studies were carried out with additional Pakistan isolates. The strains selected for detailed study represent all of the variants detected in previous studies. Because (1) of the previous demonstration of large numbers of extraneous agents in most egg seeds of SF group strains and (2) the possibility that field isolates, especially from pools of ticks, might represent mixtures of SF rickettsial strains, all strains selected for detailed study, including the strains of established reference organisms, were plaque-purified. This consisted of 3 serial plaque isolations in SPAFAS derived CE cell monolayers followed by (1) amplification and seed production in CE cells (SPAFAS), (2) production of L-929 cell seed and (3) production of yolk sac seed in SPAFAS eggs. All studies on plaque and growth characteristics, antibiotic sensitivity and slime layer production, as well as production of antisera, were performed with the plaque-purified strains. Because of the enormous amount of time involved in plaque purification and seed production, some of the preliminary serological studies reported below employed antigens already on hand which had been prepared from yolk sacs which had been inoculated with the conventional seeds prior to the time that plaque purified seeds were available. Final studies will be done exclusively with reagents prepared from plaque-purified seeds.

2. Slime layer or capsule on SF group strains. Following initial demonstration of a substantial layer exterior to the cell envelope in R. prowazeki which was removed by usual laboratory manipulation but which could be preserved to varying degrees by gentle rupture of infected cells in the presence of specific immune serum, it was demonstrated that R. rickettsii had a similar slime layer. Accordingly, all of the SF strains selected for detailed study were examined for the presence of a similar structure. The results to date are summarized in Table 3. All strains (13) on which satisfactory observations have been made show this structure. Observations on 3 strains are not adequate because of technical problems which can be resolved. However, there is some question about R. akari, but even here, it is possible that the stabilizing antiserum was of insufficient potency. Although not complete, these studies show that most SF strains, both prototype and recent isolates, elaborate an easily lost slime layer of antigenic material external to the cell envelope.

Table 2 . Spotted Fever Group Rickettsiae Employed in Current Study.

Organism	Strain	Plaque Purified	Comment
R. rickettsii	Sheila Smith	+	
R. conori	Casablanca	+	
Indian TT		+	
R. sibirica	246	+	
R. sibirica	Netz	+	
R. australis	Phillips	+	
R. akari	Hartford	+	
R. parkeri	C	+	
Pak 3358		+	*R. sibirica* - like Type 1
Pak JC 65		+	*R. sibirica* - like Type 2
Pak JC 480		+	*R. conori* - like
Pak JD 112		+	putative new species
Thai TT		+	putative new species
Czec D		+	putative new species
Israel TT-1		+	putative new species

3. Plaque characteristics. Early studies by others with one or two SF rickettsiae indicated that these SF group organisms produced larger plaques more rapidly in CE cells than rickettsiae of the typhus, scrub typhus or Q fever groups. Without adequate documentation and without the use of plaque purified materials, it has been suggested that this may be a group characteristic. If verified, this phenomenon may have considerable practical application. Accordingly, a systematic study of SF group plaque characteristics was performed.

a. Selection of standard conditions for plaque-formation. Using R. rickettsii as the SF group prototype and primary CE cells (SPAFAS) in 60 mm plastic petri dishes with 5% fetal calf serum in half-strength Dulbecco's medium with agarose as overlay medium, 32 C incubation temperature and a 5% CO_2 - 95% air atmosphere, preliminary studies were performed to establish standard conditions for comparison of strains. Plaques were counted 24 h after addition of the neutral red agarose medium overlay. Plaques could not be identified prior to 5 days after inoculation. (At about this time, plaques not visible by direct lighting could be detected by oblique-quasi-darkfield-lighting.) Both number and size of plaques increased rapidly from 5 to 7 days after inoculation after which the rate of change rapidly decline, though size continued to increase somewhat through 9-10 days. The standard time for comparison was chosen as 7 days after inoculation. (Plaque size was determined by measurement of photographs of the plaques taken at the desired time.)

b. Plaque characteristics of SF group strains under standard conditions. When the plaque size of the 14 plaque purified strains was determined under the established standard conditions, it was found that most strains produced plaques between about 1.0 and 1.2 mm in diameter. Large plaque size indeed appears to be a characteristic of most SF group strains. Two strains, however, produced plaques which were smaller than the plaques of other SF group strains - viz., R. akari and R. australis. When incubated longer than the standard 7 days, the size of the plaques continued to increase and approached that of the reference R. rickettsii whose plaque size was approaching a plateau during this interval (Table 3). It is possible that this delay in plaque size may be a function of a lag phase peculiar to the particular seeds employed.

Thus, the majority of the SF group strains do indeed produce large plaques at 7 days and this approaches a group characteristic. However, if the smaller size of the plaques of R. akari and R. australis is confirmed, this may constitute a clue to both differences of practical and basic biologic significance.

c. Search for plaque-size variants. Examination of dishes containing plaques of different SF group strains revealed a range of plaque size, from small to very large. Two alternative explanations are possible: (1) plaque size has a continuous range of distribution between wide limits or (2) spontaneous plaque size variants exist in the seeds. Plaque size has proven to be a very useful marker, sometimes associated with major differences in biological properties, among viruses. Hence, it was of both practical and theoretical importance to determine if true plaque size variants could be identified among SF group rickettsiae.

Preliminary analysis, not yet complete, of data suggests that we have not yet identified plaque size mutants. First, histograms of size distribution reveal only a single mode. Bimodal distribution curves have not yet been identified. Secondly, the distribution of plaque size of the progeny of clones of rickettsiae from large and small plaques approach that of the parent strain and do not yield true-breeding lines of small and large plaque variants.

Table 3 . Summary of Some Properties of Spotted Fever Group Rickettsiae.

Organism	Capsule/ Slime Layer[1]	Growth Characteristics[2]: Plaque Size (mm)	Infect. Cycle Type[3]	Generation Time[4] (n)	Antibiotic Action: % Plaque[5] Reduction Doxycycline) (0.1 μg/ml)	Spheroplast Formation (Pen G)
R. rickettsii (ss)	+	1.09	2	10	100	+
R. conori	+	1.09	2	15	100	+
Indian TT	+	1.22	2	9.8	100	+
R. sibirica (246)	+	0.95	2	14	100	+
R. sibirica (Netz)	N.D.	1.21	2	9.4		N.D.
R. australis	+	0.66	2	8.5	100	+
R. akari (H & fd) Hartford	Inc.	0.83	2	9.2	100	+
R. parkeri	+	1.25	2	11.5	100	+
Pak 3358	+	(1.34)	2	11.1	100	+
Pak JC 65	+	1.19	2	8.3	100	+
Pak JC 480	+	1.06	2	13.5	100	+
Pak JD 112	+	1.22	2	15.6	100	+
Thai TT	+	1.00	2	12.7	100	+
Czec D	+	1.22	2	10.2	100	+
Israeli TT-1	+	1.08	2	12.2	100	+
R. prowazeki (Breinl)	+		1	10.7	100	+
R. prowazeki (E)	+		1	10.7	100	+
R. mooseri (Wilmington)	N.D.		2	9.8	N.D.	N.D.
R. canada	N.D.		N.D.	N.D.	N.D.	N.D.

FOOTNOTES TO TABLE 3

1. Electron microscope examination of ultrathin sections of organisms grown in CE cells and gently released with immunological stabilization of slime layer.

2. In CE cells at 32 C.

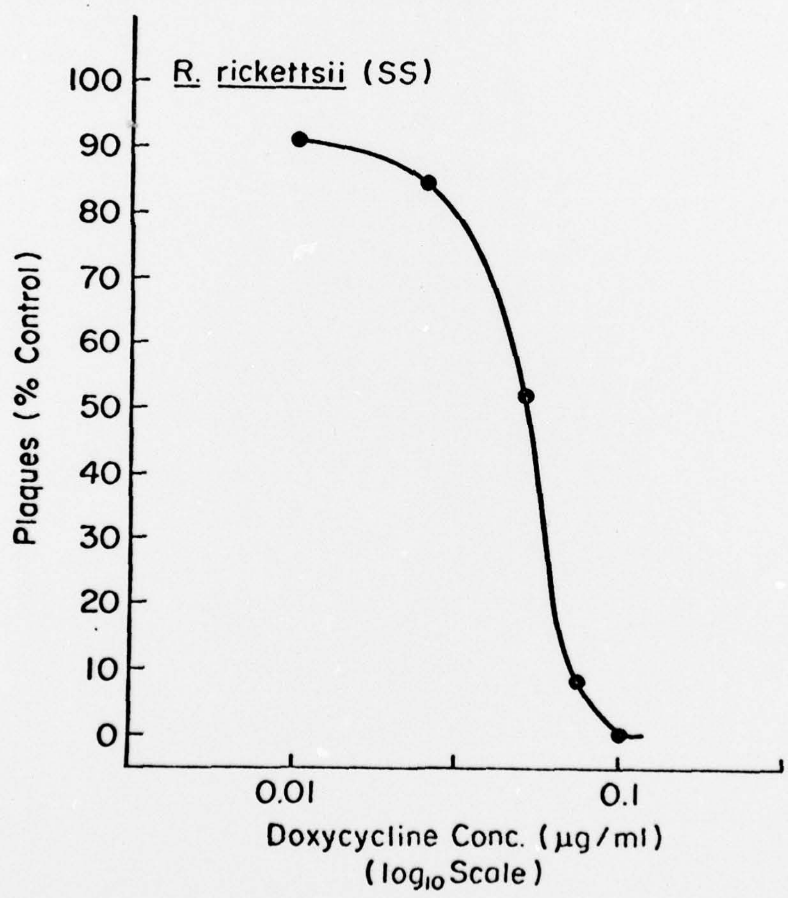
3. Infection cycle type. Type 1 = *R. prowazeki* - type of infection cycle. Type 2 ≑ spreading *R. rickettsii* type of infection cycle.

4. For spotted fever group rickettsiae and *R. mooseri*, g was calculated from average no. rickettsiae per cell. Because of abberations introduced by spreading type of infection, the values are probably significantly greater than true division time.

5. Per cent plaque reduction in presence of 0.1 µg/ml doxycycline.

6. Microscopic evidence of spheroplast formation at 100 and 500 µg/ml penicillin G at 4 and 24 h.

Figure 4. Plaque-reduction curve of R. rickettsii (Sheila Smith strain) with doxycycline.



4. Infection cycle studies. In contrast to the infection cycle described for R. prowazeki (2b) in which rickettsiae upon entering into host cell cytoplasm multiplied without significant escape until the host cell became packed with rickettsiae and broke down, R. rickettsii was found to begin to escape from the infected host cell very soon after infection, causing a rapidly spreading infection (2c). R. prowzeki thus exhibited a unidirectional passage through host cell plasma membrane, from outside in, whereas R. rickettsii exhibited bidirectional passage - i.e., from outside in and from inside out. It also had the capacity, on occasion, to penetrate into the host cell nucleus. The infection cycle and type of infection in cell cultures, thus, show marked differences between R. prowazeki and R. rickettsii, which probably reflect differences in capacity to interact with different components in the outer and inner surfaces of the host cell plasma membrane. This is probably of very great basic significance. For convenience, we have designated the infection cycle exhibited by R. prowazeki as Type 1 and that by R. rickettsii as Type 2.

We examined the infection cycle of the other members of the SF groups selected for detailed study to determine if the Type 2 infection cycle is a group characteristic. This was found to be the case (see Table 3). Thus, all SF group rickettsiae studied show the spreading type of infection. With the early escape of organisms from infected cells, calculation of accurate generation times is impossible. However, by using average number of rickettsiae per cell instead of per infected cell, an approximation representing an upper limit is possible and appears to be in the range of 10-12 hours for most of the organisms. This is not much different from 9-10 h measured for R. prowazeki, especially since it errs on the longside.

Ultrastructural studies of infected cells, in progress, show that R. rickettsii seems to have an unique action on intracellular membrane structures probably endoplasmic reticulum, not seen in cells similarly infected with R. prowazeki. It is not yet known if other members of the SF group cause similar changes in endoplasmic reticulum of host cells.

Although Type 2 infection cycle seems to be a characteristic of all SF group rickettsiae studied to date, Type 1 infection cycle is not a characteristic of the entire typhus group. Preliminary studies with R. mooseri (Wilmington strain) indicates that it exhibits a Type 2 infection cycle (Table 3). Recent, wild isolates of R. prowazeki and R. mooseri are being prepared to see if Type 1 is unique to R. prowazeki.

5. Sensitivity to antibiotics. A full-scale plaque reduction determination of the sensitivity of R. rickettsii to doxycycline revealed a curve similar to that of R. prowazeki with a similar MIC of ≤ 0.1 µg/ml. All strains showed complete suppression of plaque formation at this concentration (Table 3).

In previous studies, it was shown that penicillin G at concentration ≥ 20 µg/ml caused the intracellular formation of unstable spheroplasts in R. prowazeki. This implied certain synthetic processes in cell envelope formation and has provided a means for liberating rickettsial DNA within host cells as well as liberating DNA for electron microscopy and other studies on genome. Accordingly, the SF group strains were screened for intracellular spheroplast formation in the presence of 100 and 500 µg/ml penicillin G. All strains produced spheroplasts (Table 3).

6. Serological studies of SF group strains. The traditional means for separating SF group strains into species has been immunological-serological differences and differences in cross-protection in vaccinated guinea pigs. Until recently, the cumbersome toxin neutralization test has been the most reliable serological test for speciation. More recently, microagglutination and indirect fluroescent antibody tests have shown some promise. Accordingly, we have begun to re-examine the Pakistan, Thai, Czechoslovakian and Israeli isolates by these techniques to help gain additional information which might permit us to determine which strains are (1) essentially the same as existing prototypes, (2) similar to, but significantly different from, prototype strains (? variants of prototype strains) or (3) sufficiently different from prototype strains to be candidates for new species. Two of these strains, the Czechoslovakian D and the Israeli TT have already been proposed as new species by the investigators who isolated them on the basis of very limited, probably inadequate, serological data.

Antisera to the different SF strains were prepared initially by a modification of the method of Burgdorfer et al. Thus, between 5×10^4 and 10^5 PFU of an L-cell seed of plaque-purified organisms were inoculated intravenously into white mice which were bled for serum 10 days later. Because of the small serum yields from mice, recently we have begun to prepare immune ascitic fluid in mice. Initially, we tried to produce ascites by the Sarcoma 180-TG technique but found that the requirement of inoculating mice with rickettsiae 10 days prior to harvesting ascitic fluid presented several serious problems. Accordinlgy, we produced ascites by i.p. injection of Fruend's adjuvant and then, when ascites were nearing the desired level, inoculated the live rickettsiae I.V. and tapped ascitic fluid beginning 10 days after infection. This produced from a few mice large quantities of immune fluid with homologous MA and FA titers similar to those of the serum. Very preliminary studies suggest that specificity is at least as good as that of mouse serum. We are now in the process of preparing good working quantities of immune ascitic fluid with all of the SF strains selected for intensive study (Table 2).

Preliminary MA studies were performed with immune mouse serum, some with inadequate homologous titers, and MA antigens prepared from yolk sacs on hand which had been inoculated with SF rickettsiae that had not yet been plaque purified (Table 4). There are admitted deficiencies in these preliminary studies. Thus, the antigens may have contained substantial quantitites of extraneous agents (described in previous reports) and the titers of some of the sera were very low. Nevertheless, the recognized prototype species (Table 2) are reasonably well differentiated from one another, as has been the case with previous toxin neutralization tests. Also, consistent with the results of previous toxin neutralization tests, Pak JD-112 and Thai TT are clearly differentiated from the prototype species and other recent isolates. Thus, evidence is building up to suggest that these two isolates represent two new species of the spotted fever group according to serological criteria.

Table 4 . Preliminary Results with MA Test on Interrelationships of SF Group Strains.

Mouse Immune Sera[1]	Washed "Somatic" Antigens[2]													
	R. rickettsii	R. parkeri	R. australis	R. akari	Pak JD 112	Thai TT	R. conori	Indian TT	Pak JC 480	Israeli TT-1	R. sibirica	Czec D	Pak JC 65	Pak 3358
R. rickettsii	32	<2	<2	2	< 2	<2	<2	<2	<2	<2	4	16	<2	<2
R. parkeri	2	128	<2	2	<2	2	32	8	2	4	4	8	2	<2
R. australis	16	<2	128	4	<2	32	16	8	4	8	8	16	2	<2
R. akari	2	2	<2	16	<2	<2	16	4	2	4	2	2	<2	<2
Pak JD 112	32	<2	<2	<2	512	32	64	16	16	32	16	32	16	<2
Thai TT	2	<2	<2	2	<2	64	8	8	4	4	8	8	4	<2
R. conori	<2	16	<2	<2	<2	<2	512	<2	<2	128	<2	<2	<2	<2
Indian TT	8	<2	<2	16	<2	8	256	256	16	16	8	16	16	<2
Pak JC 480	4	<2	<2	16	<2	<2	8	16	16	8	4	8	4	<2
Israel TT-1	2	<2	<2	4	<2	16	32	32	4	64	2	32	8	<2
R. sibirica	16	4	<2	<2	32	16	32	16	16	32	64	32	32	<2
Czec D	<2	<2	<2	<2	4	4	16	8	2	<2	<2	8	8	<2
Pak JC 65	<2	<2	<2	<2	<2	<2	8	<2	<2	2	<2	8	16	<2
Pak 3358	2	<2	<2	2	<2	4	16	8	8	4	8	8	4	32

1. Produced by IV inoculation of 10^4 - 10^5 PFU of L-cell seeds of plaque purified strains.

2. Prepared from yolk sacs infected with non-plaque purified strains.

The others are more complex (Table 4). Only tentative, limited conclusions are possible at this time. On the basis of previous toxin neutralization tests, Pak JC-65, Pak 3358 and Czec D strains were found to be related to Soviet strains of R. sibirica. However, while Pak 3358 appeared closely related to, or identical with, Soviet strains by toxin neut, Pak JC 65 and Czec D (and Czec B) strains were very similar to each other but exhibited only a 1-way cross reaction with Soviet strains and Pak 3358. These preliminary MA tests, with unfortunately low titered sera, confirm the close relationship between Pak JC 65 and Czec D and their difference from Soviet strains and Pak 3358. They do not confirm yet the close relationship between the Soviet strains and Pak 3358.

Similarly, Indian TT and Pak JC 480 have been found in previous studies to be related to R. conori but on the basis of vaccination-cross challenge studies, Elisberg et al. found differences between prototype R. conori and Indian TT, confirmed by the present preliminary MA tests. Likewise, the Israeli TT-1 is similar but different.

It is possible that we are encountering strain variation in R. sibirica and R. conori complexes. But it is also possible that the differences are great enough to warrant separate designation as species, as has been applied in the SF group. Further studies employing different techniques with reagents prepared from plaque purified strains will be required to settle these problems. The results have (1) basic biological, (2) epidemiological, ecological and zoogeographical and (3) practical preventive and diagnostic implications. It is possible that there has been strain variation among major complexes (such as the putative R. conori and R. sibirica complexes) in different geographic regions where different vertebrate and arthropod hosts have exerted different selective conditions. Answers to this question would shed much light on the evolution of SF group rickettsiae. More important from a practical point of view is the influence of strain variations on diagnostic tests and on the problem of immunoprophylaxis. If antigenic drift can be established and its impact on immunity can be established, then we would have powerful tools at hand for predicting vaccine requirements in different geographic regions.

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